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Department of Biochemistry and Biophysics, Texas A and M University, 2128 TAMU, College Station, TX 77843-2128

Telomerase is a ribonucleoprotein (RNP) reverse transcriptase whose essential RNA subunit (TER) functions as a template for telomere repeat synthesis. Here we report the identification of two divergent TER moieties in the flowering plant *Arabidopsis thaliana*. Although both TER1 and TER2 copurify with telomerase activity and serve as templates for telomerase in vitro, depletion of TER1, but not TER2, leads to decreased telomerase activity and progressive telomere shortening in vivo. Moreover, mutation of the templating domain in TER1 results in the incorporation of mutant telomere repeats on chromosome ends. Thus, TER1 provides the major template for telomerase in vivo. We also show that POT1a binds TER1 with a  $K_d$  of  $2 \times 10^{-7}$  M and the two components assemble into an enzymatically active RNP in vivo. In contrast, TER1-POT1b and TER2-POT1a associations were not observed. In other organisms POT1 proteins bind telomeric DNA and provide chromosome end protection. We propose that duplication of TER and POT1 in *Arabidopsis* fueled the evolution of novel protein-nucleic acid interactions and the migration of POT1 from the telomere to the telomerase RNP.

The telomerase reverse transcriptase is responsible for synthesizing and maintaining tracts of telomeric DNA on chromosome ends. A ribonucleoprotein (RNP), telomerase contains two essential components: a catalytic reverse transcriptase subunit (TERT), and an RNA subunit (TER) that serves as a template for telomeric DNA addition. Telomerase activity is primarily confined to cells with extended proliferation potential (e.g., the germ line and stem cell populations) (1), and in settings defined by restricted proliferation programs, the enzyme is inactive. The absence of telomerase leads to progressive telomere loss, ultimately eliciting a DNA damage response that culminates in cell cycle arrest, genome instability, and, in humans, replicative cell senescence and apoptosis (2).

Telomerase access to the chromosome terminus is controlled *in cis* by telomere binding proteins. One key telomere protein is Protection Of Telomeres (POT1). POT1 binds single-strand G-rich telomeric DNA via oligonucleotide/oligosaccharide binding folds (OB folds) (3–5) that specifically exclude RNA (6). POT1 is implicated in many facets of telomere biology, including control of telomere replication (both positive and negative regulation of telomerase), suppression of the DNA damage response, and protection of chromosome ends from inappropriate recombination, nuclease attack, and end-to-end fusion (7, 8). Notably, *Arabidopsis thaliana* encodes two highly divergent POT1-like paralogs, AtPOT1a and AtPOT1b (9), neither of which binds to telomeric DNA *in vitro* (10, 11). AtPOT1a is not required for chromosome end protection. Rather, it physically associates with the telomerase RNP and serves as a positive regulator of telomerase activity *in vivo* (11).

templating domain typically corresponding to one and a half telomeric repeats flanked by a 5' boundary element and a 3' pseudoknot domain (16–19). Vertebrate TERs harbor a box H/ACA snoRNA motif, which binds dyskerin and is required for RNP maturation and nuclear localization (20).

Telomerase activity can be reconstituted in vitro with TERT and TER (16, 21, 22), although additional proteins assemble with the core telomerase RNP in vivo. For example, Est1 is a noncatalytic component of the yeast telomerase that facilitates enzyme recruitment/activation at chromosome ends (23, 24). Relocation of the Est1 binding site to a different position in TER does not diminish telomerase activity in vivo (25), indicating that TER is a modular, highly flexible scaffold for telomerase proteins. The Ku70/80 heterodimer is reported to bind TER in budding yeast and vertebrates (26, 27), but not in fission yeast (15). Ku promotes telomerase recruitment to nontelomeric DNA for de novo telomere formation in *Saccharomyces cerevisiae* (28); its role in vertebrate telomere maintenance is unknown.

We report the discovery of two distinct TER subunits in *A. thaliana*. Both TER1 and TER2 function as templates for TERT in vitro, but only TER1 plays a significant role in telomere maintenance in vivo. Moreover, we show that TER1, but not TER2, specifically binds POT1a in vitro and the two components assemble into an enzymatically active RNP in vivo. These findings underscore the evolution of telomerase and telomere proteins in Arabidopsis and argue that the process is driven by gene duplication.

**Identification of Two TERs in Arabidopsis.** We took a biochemical approach to identify the Arabidopsis TER. Telomerase was partially purified from cell culture nuclear extracts by three sequential chromatographic steps (Fig. S1). Enzyme activity was monitored after each step by the telomere repeat amplification protocol (TRAP). Size exclusion chromatography revealed that the peak of telomerase activity correlated with a complex of ~670 kDa, similar to human telomerase (29). Total RNA was extracted from heparin column peak activity fractions, 3' end radio-labeled, and separated on a 6% acrylamide gel. Numerous RNAs were detected (Fig. S2B). The gel was divided into slices, RNA was extracted, and cDNA was generated by reverse transcription using random pentadecamers. Sequencing reactions were performed with primers corresponding to the seven permu-

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tations of the Arabidopsis telomere repeat sequence ( $T_3AG_3$ )<sub>n</sub>. BLAST searches of resulting sequences retrieved a hit termed *TER1*, which maps to chromosome 1 in a region overlapping the 5' UTR and the first two exons and introns of an unknown protein coding gene, AT1G71310. *TER1* (GenBank accession no. HQ401284) encodes a putative template sequence (5'-CTAAA-CCCTA-3') corresponding to 1.5 copies of the Arabidopsis telomere repeat (Fig. 1A).

Unexpectedly, a second BLAST hit, termed *TER2* (GenBank accession no. HQ401285), was also uncovered (Fig. 1A). *TER2* maps to chromosome 5, in the opposite direction and partially overlapping with the 5' UTR of another unknown protein coding gene, AT5G24670. *TER2* also contains 1.5 copies of the Arabidopsis telomere repeat. RT-PCR confirmed that both *TER1* and *TER2* RNAs were enriched in purified telomerase fractions (Fig. S2 C and D), in contrast to a U6 snRNA control (Fig. S2 C and D). Mapping of the 5' and 3' ends revealed that *TER1* encodes a 748-nt RNA and *TER2*, a 784-nt RNA (Fig. 1A and B and Fig. S3). Unlike human (30) and yeast *TER* (31), we found no evidence of a poly-A tail for either Arabidopsis RNA.

*TER1* and *TER2* contain a 220-nt stretch of ~90% identity (Fig. 1A), but in *TER2* this segment is divided into two regions (R1 and R2) separated by an unrelated sequence of 529 nt. The 5' region (R1) in *TER2*, which corresponds to 114 nt and includes the putative telomere template, shares 85% identity with *TER1*. The downstream 75-nt block (R2) exhibits 96% identity with *TER1*. The template domain is located in different positions

in the two RNAs. In *TER1* it is embedded in the RNA (241 nt from the 5' end) similar to the human *TER*, whereas in *TER2* it lies at the extreme 5' end of the RNA (8 nt from the terminus) as in mouse *TER* (32) (Fig. 1A). Putative H/ACA boxes are present in the 3' ends of both *TER1* and *TER2*, consistent with our previous finding that dyskerin is a component of Arabidopsis telomerase (33).

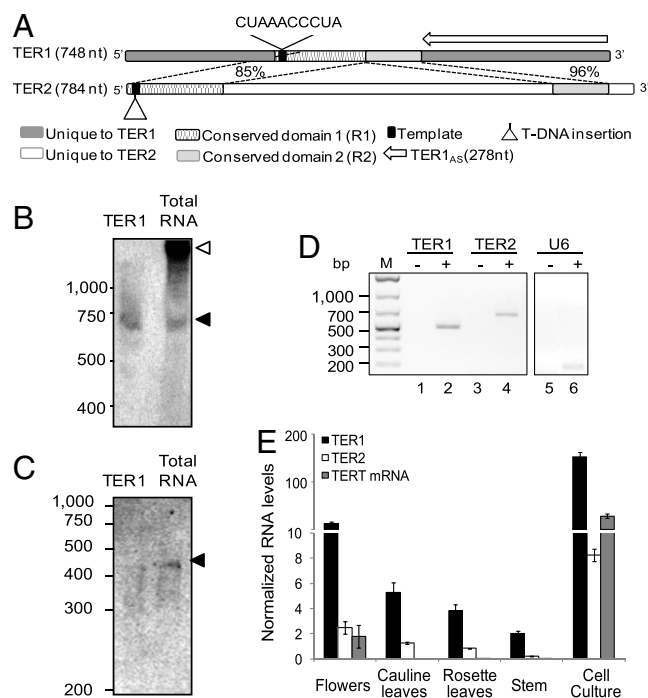
Northern blotting of total cell culture RNA revealed ~750-nt transcript corresponding to *TER1* (Fig. 1B). Primer extension confirmed the presence of a *TER1* transcript (Fig. 1C). Attempts to detect *TER2* by Northern blotting or primer extension using probes that target the conserved region in the two RNAs or the unique region in *TER2* were unsuccessful, likely due to the low abundance of this RNA. As an alternative approach, endpoint RT-PCR was employed. Both *TER1* and *TER2* were detected by this method (Fig. 1D). Quantification by real time RT-PCR showed that like *TERT* (34), *TER1* and *TER2* peak in cells with high telomerase activity (e.g., flowers and cell culture) (Fig. 1E). However, *TER1* levels were consistently higher than *TER2*. In flowers, *TER1* abundance was 10-fold higher than *TER2*, and in cell culture the difference was 20-fold (Fig. 1E).

**Both *TER1* and *TER2* Serve as Templates for Telomerase in Vitro.** To test whether *TER1* or *TER2* form a functional telomerase RNP with *TERT* in vitro, T7-tagged *TERT* was expressed in rabbit reticulocyte lysate (RRL) and incubated with in vitro transcribed *TER1* or *TER2*. RNP complexes were immunoprecipitated (IP) and telomerase activity was monitored by TRAP (Fig. 2A). Telomerase activity was dependent on the addition of *TER1* or *TER2* (Fig. 2A and B). Activity was not detected with an antisense version of *TER1* or an unrelated RNA (RNA-415) containing a 10-nt telomeric sequence (Fig. 2B). In addition, telomerase activity was not observed in the absence of *TERT* or a boiled sample control (Fig. 2B). We conclude that both *TER1* and *TER2* assemble into enzymatically active telomerase RNPs in vitro.

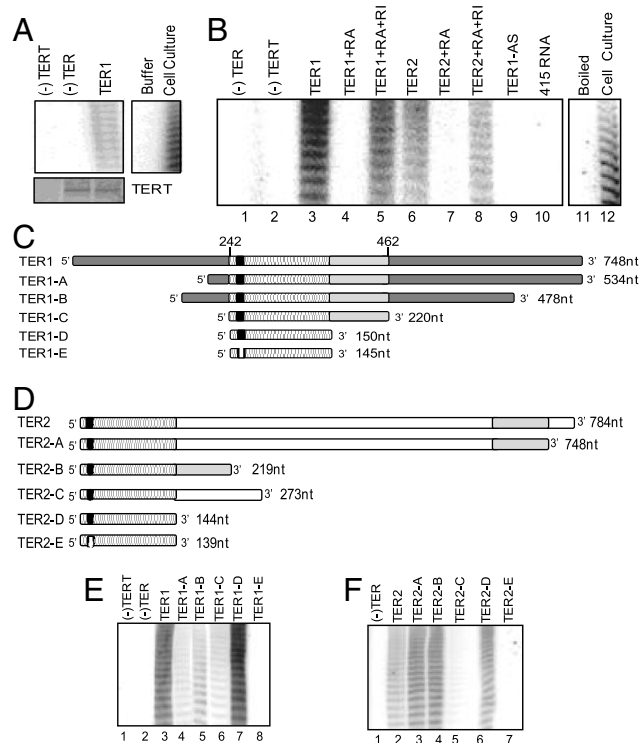
Deletional mutagenesis was used to establish the minimal *TER1* and *TER2* sequences required for telomerase activity. For *TER1*, telomerase activity was detected from in vitro assembled RNPs containing a fragment of 534 nt (*TER1*-A, Fig. 2C and E) or 478 nt (*TER1*-B, Fig. 2C and E). Notably, the 220-nt conserved region of *TER1* was sufficient to reconstitute telomerase activity (*TER1*-C, Fig. 2C and E). A minimal *TER1* of 150 nt, roughly corresponding to R1 in *TER2*, produced activity comparable to full-length *TER1* (*TER1*-D, Fig. 2C and E). As expected, no activity was recovered when a 5-nt deletion in the *TER1* template region was introduced (*TER1*-E, Fig. 2C and E).

Fusion of the two conserved segments in *TER2* (R1 and R2) to generate a contiguous 220-nt segment (*TER2*-B) reconstituted telomerase activity to the level of full-length *TER2* (Fig. 2D and F). As with *TER1*, the R1 segment in *TER2* was sufficient to fully reconstitute telomerase activity (*TER2*-D, Fig. 2D and F). However, telomerase activity was not observed when *TER2* R1 plus 129 nt of downstream sequence was expressed (*TER2*-C, Fig. 2D and F). This additional RNA segment may interfere with the folding of functional domains within *TER2* R1. Thus, the core elements required for *TER1* and *TER2* function reside within a 150-nt region conserved in both RNAs.

***TER1* Is the Template for Telomere Maintenance in Vivo.** A T-DNA insertion line (*ter1-1*) is available for *TER1*, but it is not a null allele as transcripts continue to be produced from this locus. As an alternative strategy to study *TER1* function in vivo, we expressed a *TER1* antisense RNA from the constitutive KU70 promoter (35) in wild-type plants, targeting a unique 278-nt region at the 3' end of *TER1* (*TER1*<sub>AS</sub>) (Fig. 1A). Relative to wild-type and untransformed siblings, *TER1* was reduced 10-fold in first generation (T1) KU :: *TER1*<sub>AS</sub> transformants and ~16-



**Fig. 1. Identification of two *TERs* in Arabidopsis.** (A) Diagram of *TER1* and *TER2*. Templates, unique and conserved domains are shown. The region in *TER1* targeted for antisense and the T-DNA insertion in *ter2-1* are indicated. Northern blot (B) and primer extension analysis (C) of total RNA from *A. thaliana* cell culture are shown. The probes for Northern blotting and primer extension were directed at unique regions in the 5' and 3' ends of *TER1*, respectively. Closed triangles, *TER1* transcript; open triangle, transcript that appears to be derived from AT1G71310, the protein encoding gene into which *TER1* is embedded. In vitro transcribed *TER1* served as a control for B and C. (D) RT-PCR analysis of total RNA from cell culture. cDNA was generated using random pentadecamers. Odd lanes show minus reverse transcriptase controls. U6 snRNA was amplified as a control (lanes 5,6). (E) Quantitative RT-PCR results for *TER1*, *TER2*, and *TERT* mRNA. RNA levels were normalized to U6 snRNA and to the efficiency of each primer pair to allow comparison (see SI Text).

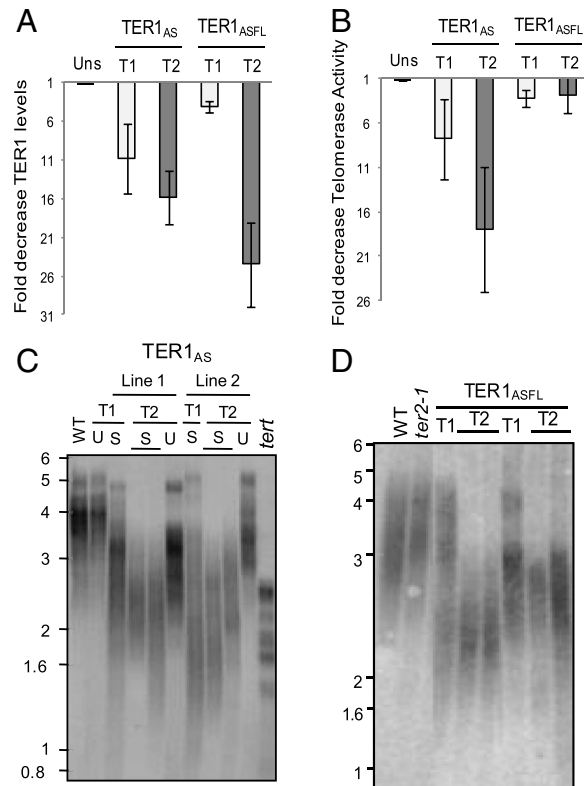


**Fig. 2. TER1 and TER2 function as templates for TERT in vitro.** (A) Telomerase reconstitution with recombinant TERT and TER1. (Top) TRAP results. A, *thaliana* cell culture, positive control; buffer, negative control. (Bottom) Western blot analysis to monitor TERT expression. (B) TRAP results with recombinant TERT, TER1, or TER2. Controls include treatment with RNase A (RA) or RNase A plus RNase Inhibitors (RI); antisense TER1 (TER1<sub>AS</sub>); unrelated *Arabidopsis* transcript containing a 10-nt sequence that could serve as putative template (415RNA); boiled reconstituted RNPs and total protein extract from cell culture. (C and D) Diagram of TER1 and TER2 deletion mutants. White block in the template indicates a 5-nt deletion. Nucleotide positions for TER1 mapping experiments (Fig. 5D) are indicated. (E and F) TRAP results for reconstitution reactions with truncated TER1 and TER2 constructs.

fold in the second generation (T2) (Fig. 3A). TER2 levels were unaffected (Fig. S4A).

Quantitative TRAP (Q-TRAP) showed that telomerase activity decreased 8-fold in T1 and 18-fold in T2 plants (Fig. 3B), paralleling the reduction in TER1. Terminal restriction fragment (TRF) analysis was performed to examine telomere length. As expected, plants transformed with the KU promoter alone or a 35S::GFP construct showed no defects in telomere maintenance (Fig. S4B). In contrast, telomere tracts were more heterogeneous and shorter in T1 KU::TER1<sub>AS</sub> mutants versus wild-type siblings (Fig. 3C and Fig. S4C). T2 KU::TER1<sub>AS</sub> plants showed preferential loss of longer telomere tracts compared to T1 transformants, and increased length homogeneity (Fig. 3C and Fig. S4C). This result reflects reduced telomerase activity, because telomerase is known to preferentially extend the shortest telomeres in the population (36). Telomeres below 1 kb are subject to end-to-end fusion in *Arabidopsis* (37). However, none of the telomeres in T2 TER1<sub>AS</sub> mutants were shorter than 1 kb, and thus it was not surprising that no defects in plant morphology, fertility, or genome instability were observed in these lines. Because the antisense mutation does not result in a complete loss of TER1, we suspect that the residual telomerase activity in these plants maintains telomeres above the critical length threshold.

Unlike TER1, there are several T-DNA insertions available for TER2. In the *ter2-1* line (SAIL\_556\_A04), the T-DNA insertion lies directly within the templating domain of TER2 (Fig. 1A). No TER2 transcript is detected in plants homozygous for *ter2-1*. In striking contrast to TER1<sub>AS</sub> mutants, we found no defects in



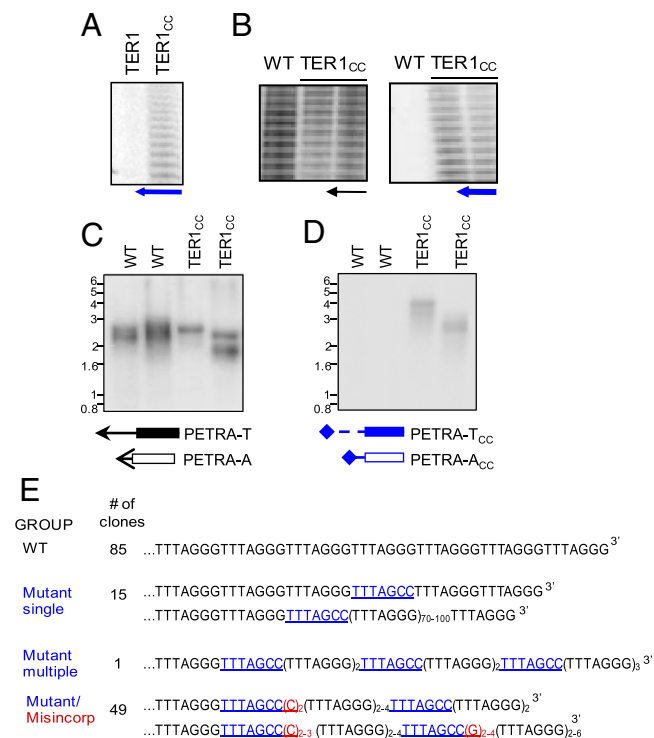
**Fig. 3. Telomere maintenance in *Arabidopsis* is dependent on TER1, but not TER2.** (A) Real time RT-PCR results for first (T1) and second (T2) generation TER1<sub>AS</sub> and TER1<sub>ASFL</sub> transformants. Results for control plants not selected on kanamycin (Uns) are included. Signals were normalized to  $\beta$ -actin and to WT TER1 levels. (B) Q-TRAP results for TER1<sub>ASFL</sub> and TER1<sub>AS</sub>. Values are normalized to telomerase activity from WT plants. (C and D) TRF analysis. In C, the T1 TER1<sub>AS</sub> plant selected for kanamycin resistance (S) is the parent of the T2 progeny. Molecular weight markers in kilobase pairs (kbp) are shown.

telomere maintenance in *ter2-1* (Fig. 3D). Simultaneous reduction of TER1 and TER2 was achieved by expressing an antisense RNA targeting full-length TER1 (35S::TER1<sub>ASFL</sub>) in a *ter2-1* background. In this setting, TER2 was undetected and TER1 was reduced 3-fold in T1 plants and 24-fold in T2 (Fig. 3A). Q-TRAP showed that telomerase activity decreased ~3-fold in both generations (Fig. 3B). T1 mutants displayed highly heterogeneous telomeres ranging from 1–6 kb (Fig. 3D and Fig. S4D). Because telomere shortening was not observed in *ter2-1* mutants and telomere length was similar in plants deficient in TER1 or both TER1/TER2, we conclude that TER1, not TER2, is required for telomere maintenance.

To examine the templating function of TER1 in vivo, site-directed mutagenesis was used to mutate the 5'-CUAAACC-CUA-3' sequence in TER1 to 5'-CUAAAGGCUA-3' (TER1<sub>CC</sub>). In vitro reconstitution reactions revealed that mutant RNA assembled into an active RNP and synthesized TTTAGCC repeats (Fig. 4A and Fig. S5A). The TER1<sub>CC</sub> construct was expressed in wild-type *Arabidopsis* under the control of the powerful cauliflower mosaic virus (CaMV) 35S promoter. As expected, TRAP products were generated when the wild-type reverse primer was used (Fig. 4B, Left, and Fig. S5A). Telomerase activity was also detected in extracts from 35S::TER1<sub>CC</sub> transformants, but not from wild type when the TER1<sub>CC</sub> reverse primer was employed (Fig. 4B, Right, and Fig. S5A).

Primer extension telomere repeat amplification (PETRA) (Fig. S5B and ref. 38) was then employed to ask if TER1 specifies the telomere repeat sequence on chromosome ends. Using a subtelomeric primer directed at the left arm of chromosome 1, PETRA products were obtained from wild-type plants and trans-





**Fig. 4.** *TER1 directs telomere repeat incorporation in vivo.* (A) In vitro reconstitution of telomerase with TER1<sub>CC</sub>. TRAP was performed with a reverse primer complementary to the mutant repeats. (B) In vivo reconstitution of telomerase with TER1<sub>CC</sub>. WT plants transformed with TER1<sub>CC</sub> were propagated for two generations and TRAP was performed with a reverse primer directed at WT (black arrow) or mutant repeats (blue arrow). (C and D) Telomere amplification by PETRA. Results for two WT plants and two third generation TER1<sub>CC</sub> transformants with either WT (black) or mutant (blue) PETRA primers. (E) Representative PETRA product sequences depicting single or multiple TTTAGCC (blue) repeats as well as misincorporation events (red) are shown.

formants expressing 35S::TER1<sub>CC</sub> (Fig. 4C). More importantly, reactions with PETRA-T<sub>CC</sub> generated products with 35S::TER1<sub>CC</sub>, but not wild-type plants (Fig. 4D).

PETRA products generated with the wild-type PETRA-T primer were cloned from 35S::TER1<sub>CC</sub> transformants. Out of 150 clones, 85 (57%) contained perfect arrays of TTTAGGG repeats, consistent with the presence of wild-type TER1 in these plants (Fig. 4E). Strikingly, 65 (43%) clones harbored one or more TTTAGCC repeats, and in 49 cases, these repeats were characterized by the misincorporation of additional C or G residues (Fig. 4E), indicating that the TER1<sub>CC</sub> mutation decreased the fidelity of telomerase in vivo. Altogether, our findings indicate that TER1 assembles into an enzymatically active RNP that determines the sequence of telomeres in vivo. The biological role of TER2 is currently under investigation, and thus the remainder of this study focuses on analysis of TER1.

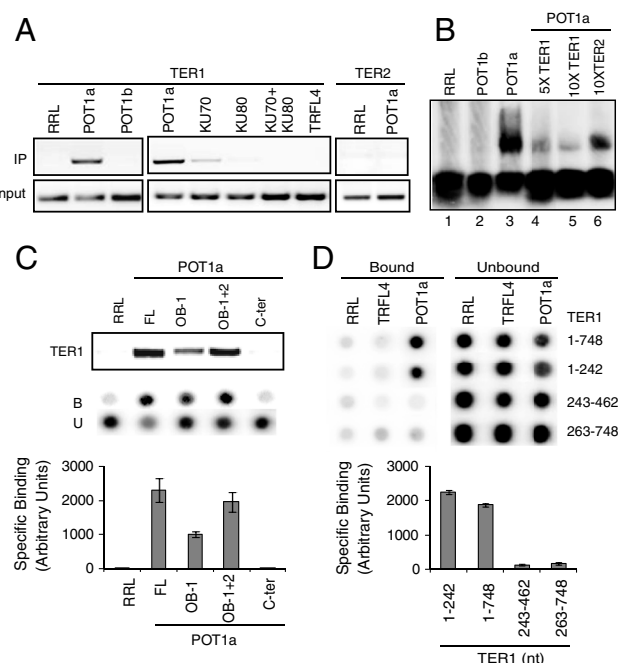
**TER1 Associates with POT1a in Vitro.** To further investigate the composition of Arabidopsis telomerase, we examined potential protein binding partners for TER1. T7-tagged KU70 or KU80 was coexpressed with TER1 in RRL (Fig. S64). IP was conducted with anti-T7 agarose beads, the bound RNA was extracted, cDNA was generated using random pentadecamers, and PCR was performed with primers specific for TER1. Only a residual amount of TER1 was detected in the KU70 or KU80 IP that was just above background with unprogrammed RRL or the IP with an unrelated protein, TRFL4 (Fig. 5A).

AtPOT1a associates with active telomerase (11), but displays only a weak in vitro interaction with dyskerin and no detectable

binding to TERT (33). Therefore, we tested whether POT1a or its paralog POT1b interacts with TERT. Tagged POT1a or POT1b was coexpressed with TERT in RRL (Fig. S64), followed by IP and PCR as discussed above. Strikingly, TERT was enriched in the POT1a IP, but not in the IP with POT1b or TRFL4 (Fig. 5A). Reactions with antisense TERT or wild-type TERT failed to show an interaction with POT1a (Fig. 5A and Fig. S7A), indicating that TERT-POT1a binding is specific. Gel shift assays produced results consistent with the IP: TERT formed an RNP complex with POT1a, but not POT1b (Fig. 5B). Competition experiments confirmed the specificity of TERT-POT1a interaction and furthermore demonstrated that POT1a does not associate with TERT *in vitro* (Fig. 5B).

Deletional mutagenesis was used to map the POT1a-TER1 interface in vitro. A combination of IP and filter binding assays were performed with recombinant POT1a and 5' end-labeled TER1 to identify the RNA binding site on POT1a (Fig. 5C). As expected, TER1 binding was dependent on the OB folds and not the C-terminal domain of POT1a. OB-1 was sufficient for TER1 binding, but a more robust interaction was observed with constructs containing both OB-1 and OB-2 (Fig. 5C). Filter binding was performed with truncated TER1 constructs to define the POT1a binding site (Fig. 5D and Fig. S6B). POT1a bound within a 5' 242-nt segment of TER1 that lies upstream of the telomere template sequence (Figs. 2C and 5D). Because this region is unique to TER1 (Fig. 1A), these results provide a possible explanation for how POT1a discriminates TER1 from TER2.

Finally, filter binding was used to measure the affinity of POT1a for TER1 *in vitro*. Recombinant POT1a was incubated with decreasing concentrations of TER1 transcribed *in vitro* with a radio-labeled tracer. The fractions of bound and free RNA were determined to calculate the  $K_d$  for the POT1a-TER1 interaction



**Fig. 5. Protein interactions with TER1.** (A) In vitro TER1 binding assays with recombinant POT1a, POT1b, and KU (Top). T7-tagged proteins were coexpressed with TER1 and RT-PCR was carried out after immunoprecipitation (IP). TRFL4, a double-strand telomeric DNA binding protein (47) served as a control. (B) Results from electrophoretic mobility shift assays. (C) Mapping the TER1 binding site on POT1a. (Top) IP results with POT1a truncation mutants; (Middle) filter binding data; (Bottom) quantitation of filter binding data. FL, full length; OB-1, first OB fold, OB-1 + 2, first and second OB folds; C-ter, C terminus; B, bound; U, unbound. (D) Mapping the POT1a binding site on TER1. (Top) Filter binding data. TER1 truncations (in nts) are indicated at Right; (Bottom) quantitation of filter binding data.

(Fig. S7B). Notably, the dissociation constant for AtPOT1a-TER1 ( $K_d = 2.1 \times 10^{-7}$  M) is similar to value obtained for purified mammalian POT1 with single-strand telomeric DNA ( $10^{-7}$ – $10^{-8}$  M) (39, 40).

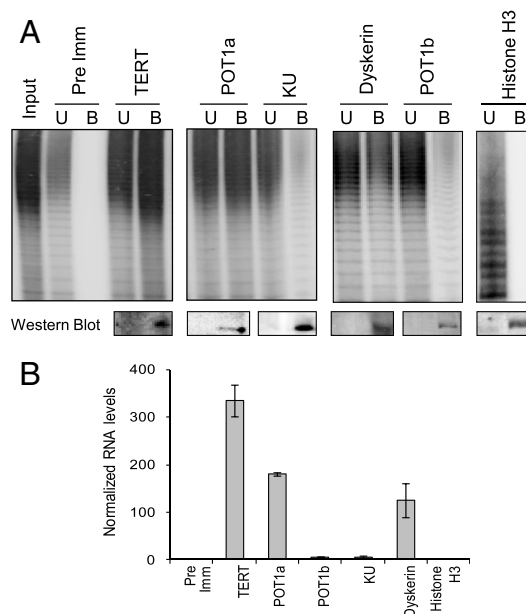
**TER1 and POT1a Assemble into an Enzymatically Active Telomerase RNP in Vivo.** We employed IP assays to investigate whether TER1 and POT1a assemble into an RNP in vivo. As expected, telomerase activity was recovered from the  $\alpha$ -TERT and  $\alpha$ -dyskerin IP samples, but not the preimmune serum or  $\alpha$ -histone H3 antibody control (Fig. 6A, Top). Western blot analysis confirmed that target proteins were enriched in their corresponding precipitates (Fig. 6A, Bottom). Attempts to quantify telomerase activity by Q-TRAP were unsuccessful, likely due to interference by the protein A agarose beads in the fluorescence intensity reading. TER1 was enriched in the  $\alpha$ -TERT and  $\alpha$ -dyskerin precipitates, but not  $\alpha$ -histone H3 (Fig. 6B and Fig. S6C). The RNA–protein interaction was specific; U6 snRNA was not detected in any of the IPs (Fig. S6C). A low level of telomerase activity was observed in  $\alpha$ -KU70 and  $\alpha$ -POT1b pull-downs (Fig. 6A), and consistent with our in vitro binding results, only a background level of TER1 was observed (Fig. 6B and Fig. S6C). In contrast, abundant telomerase activity and TER1 was detected in the  $\alpha$ -POT1a IP (Fig. 6A and B and Fig. S6C). We conclude that TER1 assembles with TERT, dyskerin, and POT1a in an enzymatically active RNP in vivo.

## Discussion

**Arabidopsis Encodes Two TERs, but Only TER1 Is Required for Telomere Maintenance in Vivo.** Of the more than 60 organisms, including yeast, ciliates, and vertebrates, where telomerase RNA subunits have been reported thus far, only Arabidopsis harbors two TER molecules encoded by distinct genetic loci. Both Arabidopsis TERs are enriched in purified telomerase fractions, both associate with TERT, and both serve as templates for telomere repeat synthesis in vitro. Indeed, a 150-nt “mini-T” constituting a region conserved in both TER1 and TER2 (R1) is sufficient to promote telomerase activity in vitro. Strikingly, this catalytic core is approximately the same size as *Tetrahymena* TER (12), and the minimal TERs defined for human (210 nt) (41) and *S. cerevisiae* (170 nt) (42). Thus, both TER1 and TER2 have the potential to function as a template for telomerase in vivo.

Despite their similarities, the Arabidopsis TERs differ dramatically in overall nucleotide sequence and protein binding partners in vivo. TER1 and TER2 are so divergent in regions flanking the conserved catalytic core that they cannot be reliably aligned. Moreover, the telomere template is located in different positions relative to the 5' terminus. Experiments with circularly permuted *Tetrahymena* TER show that the template along with a few adjacent 3' residues can be moved to different sites without affecting enzyme properties in vitro (43). Thus, the different location of the templating domains in TER1 and TER2 may have little impact on their function in vivo. On the other hand, the unique sequences in TER1 and TER2 support distinct protein binding partners. POT1a, but not POT1b, specifically associates with TER1 and not TER2. Thus, the Arabidopsis TERs, like their counterparts in other eukaryotes, are modular and flexible frameworks for protein interactions. Finally, we found that TER1 functions as a canonical telomerase RNA subunit to promote telomere maintenance in vivo, whereas TER2 does not play a significant role in this capacity. One intriguing, but untested, possibility is that TER2 lacks the binding sites for RNP components that facilitate telomerase association with chromosome ends.

**POT1a Is a TER1 Binding Protein.** Identification of the templating RNA for Arabidopsis telomerase provided insight into the protein composition of this RNP. We found that TER1 associates with both TERT and dyskerin in vivo, suggesting that like human



**Fig. 6. TER1 interactions in vivo.** Immunoprecipitation was carried out with *Arabidopsis* cell culture extracts using the indicated antibodies. Preimmune serum, and antihistone H3 antibodies were used as negative controls. (A, Top) TRAP results with unbound (U) and bound (B) IP material. (Bottom) Western blot analysis of IP fractions. (B) Quantitative RT-PCR results following IP. RNA levels were normalized to the primer efficiency, the levels of U6 snRNA, the preimmune control and the antibody efficiency.

telomerase (29), the core telomerase RNP in Arabidopsis is comprised of TERT, TER, and dyskerin. We did not detect a robust interaction between Ku and TER1. This observation is consistent with a role for TER1 in promoting telomere replication, because Arabidopsis Ku is a potent negative regulator of telomere length (35).

Our work also sheds light on AtPOT1a, a hitherto enigmatic telomeric protein. Repeated attempts to detect DNA binding by POT1 proteins from Arabidopsis and other related plant species have been unsuccessful (11, 44), indicating that POT1 likely evolved to bind a different substrate than telomeric DNA in higher plants. AtPOT1a is not required for chromosome end protection and instead physically interacts with telomerase, acting in the same genetic pathway as TERT (9, 11). Here we show that POT1a associates with telomerase via a direct interaction with TER1.

The migration of POT1 from the telomere to telomerase appears to be a relatively recent event. POT1 from algae, moss, maize, and Asparagales binds telomeric DNA in vitro (10, 45). Moreover, moss POT1, like its counterparts in yeast and vertebrates, is required for chromosome end protection (45). Our data suggest that the first OB fold in AtPOT1a provides the major contacts for TER1 recognition. Similarly, the crystal structure of human POT1 reveals several residues in OB-1 that lie in close proximity to telomeric DNA (3). One of these, F62, plays a critical role in distinguishing DNA from RNA binding (6). Intriguingly, the corresponding residue is conserved in AtPOT1a, implying that the substrate for POT1a is not simply telomeric RNA, a conclusion supported by our binding studies that show POT1a recognizes a unique region of TER1 upstream of the templating domain. Finally, the  $K_d$  we obtained for the AtPOT1a-TER1 interaction is similar to the dissociation constant of purified mammalian POT1 with single-strand telomeric DNA (39, 40). Thus, the switch from telomeric DNA to TER may reflect subtle remodeling of the nucleic acid binding pocket in POT1 and involve coevolution of POT1a with TER1.

**Duplication of Telomerase Subunits.** Duplication of telomerase components has driven diversification of function in other eukaryotes. An instructive example is found in the ciliate *Euplotes crassus*, which encodes three differentially expressed TERT isoforms (46). These TERTs assemble with a single TER into distinct RNPs that are postulated to promote a switch in telomerase specificity as the enzyme transitions from de novo telomere formation to maintenance of preexisting telomeric DNA tracts. The duplication of TER in *Arabidopsis* has likewise led to diversification in function, although the specific role of TER2 is not yet understood. POT1 proteins are also duplicated in *Arabidopsis*, and we have now shown that one of these duplicates, POT1a, is associated with the telomerase RNP through interaction with TER1. Altogether, our data indicate that gene duplication provided the necessary components for assembly of distinct RNPs in *Arabidopsis*, potentially fueling the emergence of unique regulatory mechanisms.

## Materials and Methods

TER1 was detected by Northern blot and primer extension of total RNA from *Arabidopsis thaliana* cell culture with  $^{32}\text{P}$  5'-end-labeled oligonucleotides. Endpoint and quantitative RT-PCR were used to detect TER1 in different plant tissues. RNA for in vitro experiments was transcribed using T7 RNA polymerase. Recombinant proteins used in the telomerase reconstitution experiments as well as binding assays were expressed using T7 coupled transcription translation rabbit reticulocyte lysate. Protein expression was monitored by the incorporation of  $^{35}\text{S}$  methionine. TRF, PETRA, and TRAP assays are discussed in *SI Text*. Protein–nucleic acid interactions were monitored by immunoprecipitation, EMSA, and filter binding assays. Detailed materials and methods are included in *SI Text*.

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